Table 4. Characteristics of Brachiocephalic Artery Plaques in the No-Treatment, Pitavastatin 0.1 mg/kg, and Pitavastatin 1.0 mg/kg Groups

	No Treatment (n=9)	Pitavastatin 0.1 mg/kg (n=10)	Pitavastatin 1.0 mg/kg (n=11)
Ruptured plaques per animal, n	3.3±0.2	3.2±0.7	1.9±0.3†
Plaque area, $\times 10^3$ μm^2	230±10	190±20	130±10*
Fibrous cap thickness, μm	1.6±0.2	1.5±0.1	2.6±0.4**
Lipid core area, %	15±3	26±2	14±2
Macrophage area, %	22±2	26±3	18±4
MCP-1 area, %	21±1	17±1	16±3

Data are mean±SEM. Data concerning ruptured plaques per animal were compared by ANOVA followed by Dunn multiple comparison tests. Other data were compared by ANOVA followed by Bonferroni multiple comparison tests. MCP-1 indicates monocyte chemoattractant protein-1.

*$P<0.05$ vs the no-treatment group; **$P<0.01$ vs the no-treatment group.

†$P<0.05$ vs the no-treatment group; data were compared by ANOVA followed by Dunnett multiple comparison tests.

40±5 [$\times 10^3$ μm^2; n=10], and 21±2 [$\times 10^3$ μm^2; n=11] for no treatment, pitavastatin 0.1 mg/kg, and pitavastatin 1 mg/kg, respectively; $P<0.01$ for no treatment versus pitavastatin 1 mg/kg; Figure VA in the online-only Data Supplement; Table 4). The serum lipid profile was comparable among the 3 groups (Table VII in the online-only Data Supplement). The cumulative effective dose of orally administered pitavastatin was ≈20 times greater than that of the dose of pitavastatin-NPs required to achieve plaque stability (28 versus 1.6 mg/kg, respectively).

Discussion

Advanced atherosclerotic plaques spontaneously rupture in the brachiocephalic arteries of ApoE$^{-/-}$ or low-density lipoprotein receptor–deficient (LDL-R$^{-/-}$) mice, and this rupture appears to represent several key histological features of ruptured human plaques, including an increase in plaque destabilization markers (eg, monocyte infiltration/activation, lipid accumulation, fibrous cap thinning) and evidence of disrupted and buried fibrous caps.[17,33,38,39] The recruitment of Ly-6C^{high} monocytes was observed during the development of aortic atherosclerosis in ApoE$^{-/-}$ mouse[31]; however, substantial proof for a decisive role of Ly-6C^{high} monocytes in plaque destabilization and rupture has been lacking. In the present study, we used this murine model and found that (1) the recruitment of inflammatory monocytes into the atherosclerotic plaque is critical for accelerating plaque destabilization and (2) the nanoparticle-mediated delivery of pitavastatin inhibits plaque destabilization and rupture by inhibiting the recruitment of inflammatory monocytes.

A recent study reported that local macrophage proliferation rather than monocyte recruitment is a major component of atherosclerosis formation.[40] In the present study, we demonstrated that the adoptive transfer of inflammatory monocytes accelerated plaque destabilization in a CCR2-dependent manner, which suggests a role of direct recruitment of inflammatory monocytes in this process. The different importance of monocyte recruitment and local macrophage proliferation between these studies may be attributable to the difference in the model, because angiotensin II infusion strongly induces MCP-1 expression in arterial walls,[41] which might reveal the importance of monocyte recruitment. There is a possibility that adoptive transfer of inflammatory monocytes indirectly enhanced local macrophage proliferation through an increase in serum monocyte-colony stimulating factor (Table II in the online-only Data Supplement) to accelerate atherosclerosis. Further study is needed to examine the relative importance of different mechanisms of monocyte/macrophage contribution to atherosclerosis in different pathological settings. Adoptive transfer of CCR2$^+$Ly-6C^{high} macrophages also increased serum levels of monocyte/macrophage chemoattractants (MCP-1, MCP-3, and MCP-5; Table II in the online-only Data Supplement), which might further accelerate monocyte/macrophage-mediated inflammation. These findings suggest that targeting CCR2$^+$Ly-6C^{high} inflammatory monocytes/macrophages with a DDS is a promising strategy to inhibit the destabilization of rupture-prone atherosclerotic plaques.

We used a PLGA nanoparticle as a phagocyte-directed DDS in the present study because PLGA is a biocompatible material that is already in clinical use. Intravenously administered FITC-NPs were rapidly taken up by circulating and splenic leukocytes, predominantly by monocytes/macrophages, and accumulated in macrophages in atherosclerotic plaques (Figure 2). An in vitro pharmacodynamics assay with FITC-NPs showed that nanoparticulation enhanced cellular uptake and retention over a 7-day period in cultured macrophages (Figure IV in the online-only Data Supplement). Weekly intravenous treatment with pitavastatin-NPs inhibited plaque destabilization and rupture associated with reduced macrophage infiltration and MCP-1 expression without affecting serum cholesterol levels. We previously reported that pitavastatin-NPs inhibited lipopolysaccharide-induced nuclear factor-κB activation in cultured macrophages.[24] Nuclear factor-κB is a central regulator of monocyte inflammatory activation and leads to the upregulation of MCP-1 and MMP expression.[42] Indeed, pitavastatin-NPs inhibited MCP-1 expression and gelatinase activity in macrophages both in vivo and in vitro. In the present study, interference with MCP-1/CCR2 signaling by the intravenous administration of 7ND-NP inhibited the recruitment of monocytes into the plaque, followed by plaque stabilization in the brachiocephalic artery (Figure 6), which confirms the pivotal role of MCP-1/CCR2 signaling–mediated monocyte migration, which makes this signaling pathway a therapeutic target to inhibit plaque destabilization. In the present study, aortic atherosclerosis was not reduced by intravenous injection of 7ND-NPs, which primarily target circulating monocytes and their microenvironment.[37] We have reported previously that aortic atherosclerosis in ApoE$^{-/-}$ mice fed a high-fat diet (without angiotensin II infusion) was attenuated by HVJ (hemagglutinating virus of Japan; Sendai virus) liposome–mediated 7ND gene transfer into hindlimb muscles, which maintains 7ND-mutant MCP-1 in the circulation.[34] Because we have previously shown that angiotensin II infusion strongly induces MCP-1 expression in smooth muscle cells in the aorta,[41] a different source of MCP-1 (macrophages in the setting of a high-fat

diet, and macrophages and smooth muscle cells in the setting of a high-fat diet and angiotensin II infusion) might affect the different therapeutic effect of 7ND plasmid on a different mode of administration, although this was beyond the scope of the present study.

Importantly, pitavastatin-NPs reduced the number of circulating Ly-6C^{high} inflammatory monocytes, which suggests that pitavastatin-NPs inhibited recruitment of Ly-6C^{high} monocytes from the sites of hematopoiesis to the circulation,[43] in addition to inhibiting recruitment from the circulation to the atherosclerotic lesions. Because pitavastatin-NPs did not affect CCR2 expression, in contrast with MCP-1 expression (Figure 4B), pitavastatin-NPs might inhibit monocyte chemotaxis by inhibiting the geranylgeranylation of RhoA, which regulates the ERM (ezrin/radixin/moesin) family of proteins.[44,45]

Pitavastatin was used in the present study because we previously found that this compound elicited the most potent inhibitory effects on HMG-CoA reductase activity in rodent liver microsomes compared with other statins (April, 2012). A prior study reported that daily oral administration of pitavastatin at 1 and 10 mg/kg, which exceeds the clinical norm and could lead to serious adverse side effects, attenuates the development of aortic atherosclerosis in ApoE$^{-/-}$ mice.[46] We thus examined whether nanoparticle-mediated pitavastatin delivery is superior to daily oral administration of pitavastatin alone for the inhibition of aortic atherosclerosis and plaque destabilization and rupture, and we found that oral daily administration of pitavastatin at 0.1 mg·kg^{-1}·d^{-1} for 28 days (cumulative dose, 0.1 mg/body) had no therapeutic effects, but administration of pitavastatin at 1.0 mg·kg^{-1}·d^{-1} (cumulative dose, 1.0 mg/body) showed significant therapeutic effects. Therefore, our nanoparticle-mediated DDS (0.05 mg of pitavastatin per body) potentiated the therapeutic efficacy of pitavastatin (by ≥20-fold). Our nanoparticle-mediated DDS may extend the usages of statin treatment while reducing potential side effects.

There are some limitations in the present study. First, we could not detect thrombus formation in the brachiocephalic artery or myocardial infarction or stroke in our murine model because mouse ruptured plaques rarely undergo thrombotic occlusion, which may reflect the difference in coagulation/fibrinolytic activity between mice and humans.[47]

Second, we could not determine the proportion of transferred macrophages that migrated into the arteries. Although a substantial proportion of transferred macrophages might be trapped in the reticuloendothelial system, such as liver and lung, we did observe increased macrophage infiltration to the brachiocephalic artery associated with upregulated monocyte/macrophage-associated serum proteins, which suggests that transferred macrophages were biologically active in the present study.

Third, the tissue concentrations of pitavastatin could not be measured because the sample size from the brachiocephalic arteries was too small for high-performance liquid chromatography. Although we examined the plasma level of pitavastatin in the pitavastatin and pitavastatin-NP groups, the concentration of pitavastatin could be measured only >2 hours after intravenous injection. Further studies are needed to clarify the in vivo pharmacokinetics of pitavastatin after pitavastatin-NP treatment in larger animals.

Finally, we only adopted the protocol of weekly intravenous administration of pitavastatin-NPs at the indicated dose. The dose of pitavastatin-NPs used in the present study was selected because of its effectiveness in previous studies from our laboratory in murine and rabbit models of hindlimb ischemia.[22,23] Further studies are needed to determine the optimal dose range and interval of pitavastatin-NPs for clinical application.

In conclusion, recruitment of inflammatory monocytes is critical in the pathogenesis of plaque destabilization and rupture. Nanoparticle-mediated pitavastatin delivery inhibited plaque destabilization and rupture and regulated the recruitment of inflammatory monocytes by interfering in MCP-1/CCR2 signaling in this model. This nanotechnology-based modality can be developed as a new therapeutic strategy for vulnerable patients with rupture-prone unstable plaques.

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Disclosures

Dr Egashira holds a patent on the results reported in the present study. The other authors report no conflicts.

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Magid D, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Nichol G, Paynter NP, Schreiner PJ, Sorlie PD, Stein J, Turan TN, Virani SS, Wong ND, Woo D, Turner MB; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics–2013 update: a report from the American Heart Association. *Circulation*. 2013;127:e6–e245.
2. Nahrendorf M, Pittet MJ, Swirski FK. Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation*. 2010;121:2437–2445.
3. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005;352:1685–1695.
4. Gordon S. Macrophage heterogeneity and tissue lipids. *J Clinical Invest*. 2007;117:89–93.
5. Tsujioka H, Imanishi T, Ikejima H, Kuroi A, Takarada S, Tanimoto T, Kitabata H, Okochi K, Arita Y, Ishibashi K, Komukai K, Kataiwa H, Nakamura N, Hirata K, Tanaka A, Akasaka T. Impact of heterogeneity of human peripheral blood monocyte subsets on myocardial salvage in patients with primary acute myocardial infarction. *J Am Coll Cardiol*. 2009;54:130–138.
6. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, Packard CJ; West of Scotland Coronary Prevention Study Group. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. *N Engl J Med*. 1995;333:1301–1307.
7. Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA, Langendorfer A, Stein EA, Kruyer W, Gotto AM Jr. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS: Air Force/Texas Coronary Atherosclerosis Prevention Study. *JAMA*. 1998;279:1615–1622.

8. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet*. 1994;344:1383–1389.
9. Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, Brown L, Warnica JW, Arnold JM, Wun CC, Davis BR, Braunwald E; Cholesterol and Recurrent Events Trial Investigators. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. *N Engl J Med*. 1996;335:1001–1009.
10. Cannon CP, Braunwald E, McCabe CH, Rader DJ, Rouleau JL, Belder R, Joyal SV, Hill KA, Pfeffer MA, Skene AM; Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 Investigators. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *N Engl J Med*. 2004;350:1495–1504.
11. Nissen SE, Tuzcu EM, Schoenhagen P, Crowe T, Sasiela WJ, Tsai J, Orazem J, Magorien RD, O'Shaughnessy C, Ganz P; Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) Investigators. Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. *N Engl J Med*. 2005;352:29–38.
12. Nicholls SJ, Ballantyne CM, Barter PJ, Chapman MJ, Erbel RM, Libby P, Raichlen JS, Uno K, Borgman M, Wolski K, Nissen SE. Effect of two intensive statin regimens on progression of coronary disease. *N Engl J Med*. 2011;365:2078–2087.
13. Patti G, Cannon CP, Murphy SA, Mega S, Pasceri V, Briguori C, Colombo A, Yun KH, Jeong MH, Kim JS, Choi D, Bozbas H, Kinoshita M, Fukuda K, Jia XW, Hara H, Cay S, Di Sciascio G. Clinical benefit of statin pretreatment in patients undergoing percutaneous coronary intervention: a collaborative patient-level meta-analysis of 13 randomized studies. *Circulation*. 2011;123:1622–1632.
14. Aikawa M, Rabkin E, Sugiyama S, Voglic SJ, Fukumoto Y, Furukawa Y, Shiomi M, Schoen FJ, Libby P. An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. *Circulation*. 2001;103:276–283.
15. Liao JK. Effects of statins on 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition beyond low-density lipoprotein cholesterol. *Am J Card*. 2005;96:24F–33F.
16. Bea F, Blessing E, Bennett B, Levitz M, Wallace EP, Rosenfeld ME. Simvastatin promotes atherosclerotic plaque stability in apoE-deficient mice independently of lipid lowering. *Arterioscler Thromb Vasc Biol*. 2002;22:1832–1837.
17. Johnson J, Carson K, Williams H, Karanam S, Newby A, Angelini G, George S, Jackson C. Plaque rupture after short periods of fat feeding in the apolipoprotein E-knockout mouse: model characterization and effects of pravastatin treatment. *Circulation*. 2005;111:1422–1430.
18. Ni W, Egashira K, Kataoka C, Kitamoto S, Koyanagi M, Inoue S, Takeshita A. Antiinflammatory and antiarteriosclerotic actions of HMG-CoA reductase inhibitors in a rat model of chronic inhibition of nitric oxide synthesis. *Circ Res*. 2001;89:415–421.
19. Egashira K, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Inou T, Takeshita A. Reduction in serum cholesterol with pravastatin improves endothelium-dependent coronary vasomotion in patients with hypercholesterolemia. *Circulation*. 1994;89:2519–2524.
20. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol*. 2001;21:1712–1719.
21. Egashira K. Clinical importance of endothelial function in arteriosclerosis and ischemic heart disease. *Circ J*. 2002;66:529–533.
22. Kubo M, Egashira K, Inoue T, Koga J, Oda S, Chen L, Nakano K, Matoba T, Kawashima Y, Hara K, Tsujimoto H, Sueishi K, Tominaga R, Sunagawa K. Therapeutic neovascularization by nanotechnology-mediated cell-selective delivery of pitavastatin into the vascular endothelium. *Arterioscler Thromb Vasc Biol*. 2009;29:796–801.
23. Oda S, Nagahama R, Nakano K, Matoba T, Kubo M, Sunagawa K, Tominaga R, Egashira K. Nanoparticle-mediated endothelial cell-selective delivery of pitavastatin induces functional collateral arteries (therapeutic arteriogenesis) in a rabbit model of chronic hind limb ischemia. *J Vasc Surg*. 2010;52:412–420.
24. Chen L, Nakano K, Kimura S, Matoba T, Iwata E, Miyagawa M, Tsujimoto H, Nagaoka K, Kishimoto J, Sunagawa K, Egashira K. Nanoparticle-mediated delivery of pitavastatin into lungs ameliorates the development and induces regression of monocrotaline-induced pulmonary artery hypertension. *Hypertension*. 2011;57:343–350.
25. Gibaud S, Demoy M, Andreux JP, Weingarten C, Gouritin B, Couvreur P. Cells involved in the capture of nanoparticles in hematopoietic organs. *J Pharm Sci*. 1996;85:944–950.
26. Sigovan M, Boussel L, Sulaiman A, Sappey-Marinier D, Alsaid H, Desbleds-Mansard C, Ibarrola D, Gamondès D, Corot C, Lancelot E, Raynaud JS, Vives V, Laclédère C, Violas X, Douek PC, Canet-Soulas E. Rapid-clearance iron nanoparticles for inflammation imaging of atherosclerotic plaque: initial experience in animal model. *Radiology*. 2009;252:401–409.
27. Ishibashi M, Egashira K, Zhao Q, Hiasa K, Ohtani K, Ihara Y, Charo IF, Kura S, Tsuzuki T, Takeshita A, Sunagawa K. Bone marrow-derived monocyte chemoattractant protein-1 receptor CCR2 is critical in angiotensin II-induced acceleration of atherosclerosis and aneurysm formation in hypercholesterolemic mice. *Arterioscler Thromb Vasc Biol*. 2004;24:e174–e178.
28. Satoh K, Nigro P, Matoba T, O'Dell MR, Cui Z, Shi X, Mohan A, Yan C, Abe J, Illig KA, Berk BC. Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms. *Nat Med*. 2009;15:649–656.
29. Ishibashi M, Hiasa K, Zhao Q, Inoue S, Ohtani K, Kitamoto S, Tsuchihashi M, Sugaya T, Charo IF, Kura S, Tsuzuki T, Ishibashi T, Takeshita A, Egashira K. Critical role of monocyte chemoattractant protein-1 receptor CCR2 on monocytes in hypertension-induced vascular inflammation and remodeling. *Circ Res*. 2004;94:1203–1210.
30. Han KH, Ryu J, Hong KH, Ko J, Pak YK, Kim JB, Park SW, Kim JJ. HMG-CoA reductase inhibition reduces monocyte CC chemokine receptor 2 expression and monocyte chemoattractant protein-1-mediated monocyte recruitment in vivo. *Circulation*. 2005;111:1439–1447.
31. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6C^{hi} monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J Clin Invest*. 2007;117:195–205.
32. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med*. 2007;204:3037–3047.
33. Gough PJ, Gomez IG, Wille PT, Raines EW. Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice. *J Clin Invest*. 2006;116:59–69.
34. Ni W, Egashira K, Kitamoto S, Kataoka C, Koyanagi M, Inoue S, Imaizumi K, Akiyama C, Nishida KI, Takeshita A. New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice. *Circulation*. 2001;103:2096–2101.
35. Inoue S, Egashira K, Ni W, Kitamoto S, Usui M, Otani K, Ishibashi M, Hiasa K, Nishida K, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E-knockout mice. *Circulation*. 2002;106:2700–2706.
36. Ni W, Kitamoto S, Ishibashi M, Usui M, Inoue S, Hiasa K, Zhao Q, Nishida K, Takeshita A, Egashira K. Monocyte chemoattractant protein-1 is an essential inflammatory mediator in angiotensin II-induced progression of established atherosclerosis in hypercholesterolemic mice. *Arterioscler Thromb Vasc Biol*. 2004;24:534–539.
37. Matoba T, Egashira K. Anti-inflammatory gene therapy for cardiovascular disease. *Curr Gene Ther*. 2011;11:442–446.
38. Rosenfeld ME, Polinsky P, Virmani R, Kauser K, Rubanyi G, Schwartz SM. Advanced atherosclerotic lesions in the innominate artery of the ApoE knockout mouse. *Arterioscler Thromb Vasc Biol*. 2000;20:2587–2592.
39. Clarke MC, Figg N, Maguire JJ, Davenport AP, Goddard M, Littlewood TD, Bennett MR. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nat Med*. 2006;12:1075–1080.
40. Robbins CS, Hilgendorf I, Weber GF, Theurl I, Iwamoto Y, Figueiredo JL, Gorbatov R, Sukhova GK, Gerhardt LM, Smyth D, Zavitz CC, Shikatani EA, Parsons M, van Rooijen N, Lin HY, Husain M, Libby P, Nahrendorf M, Weissleder R, Swirski FK. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med*. 2013;19:1166–1172.
41. Koga J, Egashira K, Matoba T, Kubo M, Ihara Y, Iwai M, Horiuchi M, Sunagawa K. Essential role of angiotensin II type 1a receptors in the host vascular wall, but not the bone marrow, in the pathogenesis of angiotensin II-induced atherosclerosis. *Hypertens Res*. 2008;31:1791–1800.
42. Mantovani A, Garlanda C, Locati M. Macrophage diversity and polarization in atherosclerosis: a question of balance. *Arterioscler Thromb Vasc Biol*. 2009;29:1419–1423.
43. Robbins CS, Chudnovskiy A, Rauch PJ, Figueiredo JL, Iwamoto Y, Gorbatov R, Etzrodt M, Weber GF, Ueno T, van Rooijen N, Mulligan-Kehoe MJ, Libby P, Nahrendorf M, Pittet MJ, Weissleder R, Swirski FK. Extramedullary hematopoiesis generates Ly-6C(high) monocytes that infiltrate atherosclerotic lesions. *Circulation*. 2012;125:364–374.

44. Wong B, Lumma WC, Smith AM, Sisko JT, Wright SD, Cai TQ. Statins suppress THP-1 cell migration and secretion of matrix metalloproteinase 9 by inhibiting geranylgeranylation. *J Leukoc Biol*. 2001;69:959–962.
45. Worthylake RA, Lemoine S, Watson JM, Burridge K. RhoA is required for monocyte tail retraction during transendothelial migration. *J Cell Biol*. 2001;154:147–160.
46. Sata M, Nishimatsu H, Osuga J, Tanaka K, Ishizaka N, Ishibashi S, Hirata Y, Nagai R. Statins augment collateral growth in response to ischemia but they do not promote cancer and atherosclerosis. *Hypertension*. 2004;43:1214–1220.
47. Matoba T, Sato K, Egashira K. Mouse models of plaque rupture. *Curr Opin Lipidol*. 2013;24:419–425.

CLINICAL PERSPECTIVE

Acute myocardial infarction is the most severe type of coronary heart disease. Recent advances in therapeutic intervention for acute myocardial infarction have been associated with an increased prevalence of heart failure with high long-term mortality, which remains a serious concern worldwide. The pathophysiological process of acute myocardial infarction includes atherosclerotic coronary plaque destabilization and rupture. In clinical settings, the use of HMG-CoA reductase inhibitors (statins) reduces cardiovascular risks; however, even a high-dose strong statin is insufficient to suppress acute myocardial infarction. In the present study, we identified circulating $CCR2^{+}Ly\text{-}6C^{high}$ inflammatory monocytes/macrophages as a culprit and a therapeutic target for plaque destabilization and rupture. We engineered poly(lactic-co-glycolic acid) (PLGA) nanoparticles containing pitavastatin, which was taken up mainly by circulating monocytes. PLGA nanoparticle–mediated delivery of pitavastatin inhibited aortic atherosclerosis and the plaque destabilization and rupture associated with decreased monocyte chemoattractant protein-1/CCR2 signaling–mediated monocyte infiltration and gelatinase activity in the plaque. A nanoparticle-mediated drug-delivery system potentiates the therapeutic efficacy of pitavastatin at least 20-fold compared with daily oral administration of pitavastatin. We are now performing a phase I/IIa clinical trial of nanoparticles encapsulated with pitavastatin in patients with critical limb ischemia (UMIN [University Hospital Medical Information Network] clinical trial registry No. UMIN000008011). Given the safety profile of GMP (good manufacturing practices)-compliant pitavastatin-encapsulated nanoparticles, future clinical trials will examine their clinical value in patients with unstable coronary plaques. Finally, nanoparticle-mediated drug delivery is a novel modality that may advance current statin treatment for unstable plaques and achieve an optimal therapeutic strategy for the prevention of acute myocardial infarction in the future.

SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Diet preparation

A high-fat diet (HFD) that contained 21% fat from lard and was supplemented with 0.15% (wt/wt) pure cholesterol (Oriental yeast, Tokyo Japan) was prepared according to the formula recommended by the American Institute of Nutrition. This diet contained the following constituents: casein, cystine, corn starch, sucrose, cholic acid, a mineral mixture, a vitamin mixture, powdered cellulose, choline bitartrate, and tert-butylhydroquinone.

Experimental protocols

At 16 to 18 weeks of age, mice began receiving the HFD. After 4 weeks of HFD, all mice were infused with angiotensin II dissolved in phosphate-buffered saline (PBS) at 1.9 mg/kg per day via an osmotic mini-pump (Alzet, Cupertino, CA, USA) for 4 weeks.[1, 2] Systolic blood pressure and heart rate were measured by the tail-cuff method and body weight was measured 4 weeks after the angiotensin II infusion. Mice were euthanized with intraperitoneal injection of pentobarbital at day 28 of angiotensin II infusion for analysis. Blood samples were collected via the left ventricles. Commercially available enzyme-linked immunosorbent assay kits (Wako Pure Chemical Industries, Osaka, Japan) were used to measure plasma lipid profiles (total cholesterol and triglycerides). Serum levels of various biomarkers were measured with the Luminex LabMAP instruments (Charles River Laboratories, Wilmington, MA, USA) (http://www.criver.com/en-US/ProdServ/ByType/Discovery/Pages/PlasmaBiomarkerAnalysis.aspx).

Experimental protocol 1: To examine whether inflammation-activated macrophages accelerate plaque destabilization and rupture, $CCR2^{+/+}$-inflammatory macrophages from the peritoneal cavity of $ApoE^{-/-}CCR2^{+/+}$ mice and $CCR2^{-/-}$-leukocytes from the peritoneal

cavity of ApoE$^{-/-}$CCR2$^{-/-}$ mice were collected three days after intraperitoneal injection of 2 ml of 0.05% thioglycollate (BD Biosciences, Franklin Lakes, NJ, USA), and these cells were directly injected intravenously three times during angiotensin II infusion. At the beginning of the angiotensin II infusion, animals were divided into 2 groups: (i) the CCR2$^{+/+}$-inflammatory macrophage group (1x10^6 cells/ 200 μl PBS) (n=5); and (ii) the CCR2$^{-/-}$-leukocyte group (1x10^6 cells/ 200 μl PBS) (n=8). Mice were euthanized over a several day period after 4 weeks of angiotensin II infusion for this protocol.

Experimental protocol 2: To examine the effect of nanoparticle-mediated delivery of pitavastatin on plaque destabilization and rupture, animals were divided into 4 groups at the beginning of angiotensin II infusion: (i) the no treatment group (n=9); (ii) the FITC-incorporated NP group (0.1 mg PLGA/ 200 μl PBS) (n=7); (iii) the pitavastatin-only group (0.012 mg pitavastatin/ 200 μl PBS) (n=6); and (iv) the pitavastatin-incorporated NP group (0.1 mg PLGA/ 0.012 mg pitavastatin/ 200 μl PBS) (n=10). FITC-NP, pitavastatin, and pitavastatin-NP were administered intravenously via the tail vein once per week.

Experimental protocol 3: To examine the effect of nanoparticle-mediated delivery of the 7ND plasmid (a dominant negative inhibitor of MCP-1[3-6]) on plaque destabilization and rupture, animals were divided into 2 groups at the beginning of the angiotensin II infusion: (i) the FITC-incorporated NP group (1.3 mg PLGA/ 200 μl PBS) (n=12); and (ii) the 7ND plasmid-incorporated NP group (1.3 mg PLGA/ 5 μg 7ND plasmid/ 200 μl PBS) (n=10). NPs were administered by weekly intravenous injection.

Experimental Protocol 4: To examine the effect of daily oral administration of pitavastatin on plaque destabilization and rupture, animals were divided into 2 groups at the beginning of angiotensin II infusion: (i) the low pitavastatin group (lower dose: 0.1 mg/kg per day); and (ii) the high pitavastatin group (higher dose: 1.0 mg/kg per day). Pitavastatin was

administered by oral gavage every day for 4 weeks.

The no treatment group in protocol 2 was also used as the control group in protocols 1 and 4.

Histopathology

To quantify the extent of the atherosclerotic lesions in the whole aorta, the aortic arch and the thoracic aorta was opened longitudinally, stained with oil red O, and pinned on a black wax surface. The percentage of the plaque area stained by oil red O with respect to the total luminal surface area was quantified. To quantify the extent of the atherosclerotic lesions in the aortic root, approximately 3 serial cross sections (5 μm thick) of the aortic root were prepared according to the method described by Paigen et al,[7] with a slight modification. In brief, atherosclerotic lesions in the aortic sinus region were examined at 3 locations, each separated by 100 μm, with the most proximal site starting after the appearance of at least two aortic valve leaflets. Serial sections were stained with elastica van Gieson (EVG). The largest plaque of the three valve leaflets was adopted for morphological analysis. The brachiocephalic arteries were embedded in paraffin or OCT compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan). Sections were cut at 3 μm for paraffin-embedded sections or 5 μm for OCT-embedded sections. Three sets of serial sections obtained at 30 μm intervals (starting from the proximal end) were stained with EVG to measure the total number of disrupted and buried fibrous caps, as previously described.[8] Disrupted and buried fibrous caps were defined as follows. A disrupted fibrous cap was defined as a visible defect in the cap accompanied by an intrusion of erythrocytes into the plaque. A buried fibrous cap was defined as an elastin layer that was overlaid with foam cells. All morphometric analyses were made on EVG-stained sections, and three vessel cross sections were quantified per mouse by computerized image analysis. Fibrous cap thickness was determined at the thinnest part of the cap by computerized image

analysis. The analysis was necessarily restricted to those plaques that had developed sufficiently to form fibrous caps, and the average thickness of three fibrous caps per section was obtained for quantitative analysis. Plaque macrophage or MCP-1 expression areas were determined by the ratio of Mac3- or MCP-1-positive areas to the intima areas of plaques.

Immunohistochemistry

Serial brachiocephalic arterial sections adjacent to those sections that were stained with EVG were deparaffinized, and endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 5 minutes. For antigen retrieval, sections were boiled for 20 minutes in citrate buffer (pH=6.0). After blocking with 3% skim milk, sections were incubated overnight at 4°C with the following antibodies: anti-mouse macrophage antibody (Mac3; dilution 1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and anti-mouse MCP-1 antibody (dilution 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by incubation with biotin-conjugated secondary antibodies. Then, the sections were washed and treated with avidin-peroxidase. The sections were developed using the DAB substrate kit (Wako Pure Chemical Industries, Osaka, Japan), and nuclei were counterstained with hematoxylin. Serial aortic root sections were also stained using anti-mouse Mac3 antibody. Multiple observers who were blinded to the experiment protocol performed the quantitative analysis. All images were captured with a Nikon microscope equipped with a digital camera (HC-2500) and analyzed using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA, USA) and Scion Image 1.62 for Windows (Scion, Frederick, MD, USA).

Flow cytometry

Peripheral blood was drawn via a cardiac puncture, and red blood cells were lysed with

VersaLyse Lysing solution (Becton Dickinson Biosciences, San Jose, California) for 10 minutes at room temperature. Spleens were removed and triturated in HBSS at 4 °C and filtered through nylon mesh (BD Biosciences). The cell suspension was centrifuged at 300 x *g* for 5 minutes at 4 °C. Red blood cells were also lysed with VersaLyse Lysing solution. After blocking the Fc receptor with anti-CD16/32 mAb (BD Pharmingen, San Diego, California) for 5 minutes at 4°C, peripheral leukocytes were incubated with a cocktail of CA, USA against CD11b-APC (BD Pharmingen, San Diego, California), CD115-PE (BD Pharmingen, San Diego, California) and Ly-6C-FITC (eBioscience, San Diego, CA, USA), and peritoneal leukocytes were incubated with a cocktail of mAb against F4/80-APC (AbD Serotec, Oxford, UK), CD115-PE (BD Pharmingen) and Ly-6C-FITC (eBioscience) for 30 minutes at 4°C; all leukocytes were then analyzed with FACSCalibur (Becton Dickinson Biosciences, San Jose, CA, USA). For the cellular uptake of FITC-NPs, leukocytes were incubated with a cocktail of mAb against lineage cell marker (Lin: CD90/B220/CD49b/NK1.1/Ly-6G)-PE (BD Pharmingen, San Diego, CA, USA) and CD11b-APC. The leukocytes were also incubated with appropriate isotype controls (BD Pharmingen, San Diego, CA, USA). Macrophage subsets were identified as either Ly-6C^{hi}F4/80^{+}CD115^{+} or Ly-6C^{lo}F4/80^{+}CD115^{+}, as previously described.[9] Monocyte subsets were identified as either Ly-6C^{hi}CD11b^{+}CD115^{+} or Ly-6C^{lo}CD11b^{+}CD115^{+}, as previously described.[10] For the cellular uptake of FITC-NPs, neutrophils and monocytes were identified as Lin^{+}CD11b^{+} and Lin^{-}CD11b^{+}, respectively.

In vivo accumulation of inflammation-activated macrophages

Thioglycollate-elicited macrophages were labeled with PKH26 (Sigma Aldrich, St. Louis, MO, USA) *ex vivo* according to the manufacturer's protocol. 24 hours after the intravenous injection of PKH26-labeled macrophages, the brachiocephalic arteries were

fixed with 3.7% formaldehyde and embedded in OCT compound. Sections were cut at 5 μm for OCT-embedded sections and evaluated by fluorescence microscopy. Nuclei were stained with DAPI (Vector Laboratories Inc., Burlingame, California).

Splenic Monocyte/Macrophage isolation and adoptive transfer

Splenic monocytes and macrophages were obtained by negative selection using the Mouse Monocytes Enrichment Kit (StemCell Technologies, British Columbia, Canada) according to the manufacturer's protocol. In brief, monocytes and macrophages were isolated from an $ApoE^{-/-}$ mouse spleen. Cells were passed through a 100 μm nylon filter, and suspended in lysis buffer. Mouse monocytic cells were further purified from the spleens using the Purple EasySep magnet (StemCell Technologies) prior to flow cytometry analysis. These cells (0.8-1.0 $x10^6$ cells/ 200 μl PBS) were directly injected intravenously via the femoral vein once to $ApoE^{-/-}$ mice fed a high-fat diet and infused with angiotensin II. Mice were euthanized with intraperitoneal injection of pentobarbital at day 7 of angiotensin II infusion for analysis.

Preparation of PLGA nanoparticles

Poly(lactic-co-glycolic acid) (PLGA) polymer with an average molecular weight of 20,000 and a lactide-to-glycolide copolymer ratio of 75:25 (Wako Pure Chemical Industries, Osaka, Japan) was used to prepare the nanoparticles. PLGA nanoparticles incorporated with fluorescein isothiocyanate (FITC; Dojindo Laboratories, Kumamoto, Japan) (FITC-NP), pitavastatin (Kowa Pharmaceutical Co Ltd, Tokyo, Japan) (pitavastatin-NP), or 7ND plasmid (7ND-NP) were prepared by a previously reported emulsion solvent diffusion method in purified water.[11-13] PLGA was dissolved in a mixture of acetone and methanol. Then, FITC, pitavastatin, or 7ND plasmid was added to this solution. The resultant PLGA-FITC, PLGA-pitavastatin, or PLGA-7ND solution was